Tannin Degradation by a Novel Tannase Enzyme Present in Some *Lactobacillus plantarum* Strains

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Lactobacillus plantarum is frequently isolated from the fermentation of plant material where tannins are abundant. *L. plantarum* strains possess tannase activity to degrade plant tannins. An *L. plantarum* tannase (TanB$_{Lp}$, formerly called TanLp1) was previously identified and biochemically characterized. In this study, we report the identification and characterization of a novel tannase (TanA$_{Lp}$). While all 29 *L. plantarum* strains analyzed in the study possess the *tanB$_{Lp}$* gene, the gene *tanA$_{Lp}$* was present in only four strains. Upon methyl gallate exposure, the expression of *tanB$_{Lp}$* was induced, whereas *tanA$_{Lp}$* expression was not affected. TanA$_{Lp}$ showed only 27% sequence identity to TanB$_{Lp}$, but the residues involved in tannase activity are conserved. Optimum activity for TanA$_{Lp}$ was observed at 30°C and pH 6 in the presence of Ca^{2+} ions. TanA$_{Lp}$ was able to hydrolyze gallate and protocatechuate esters with a short aliphatic alcohol substituent. Moreover, TanA$_{Lp}$ was able to fully hydrolyze complex gallotannins, such as tannic acid. The presence of the extracellular TanA$_{Lp}$ tannase in some *L. plantarum* strains provides them an advantage for the initial degradation of complex tannins present in plant environments.

Tannins are present in a variety of plants that are utilized as food and feed (1). Tannins seem to be a two-edged sword, since they are beneficial to health due to their chemopreventive activities against carcinogenesis and mutagenesis, but simultaneously, they may be involved in cancer formation, hepatotoxicity, or antinutritional activity (2). The molar mass of tannin molecules affects the tannin characteristics directly. It has been found that the higher the molar mass of tannin molecules, the stronger the antinutritional effects and the lower the biological activities (2). Small-molecule tannins are suggested to have fewer antinutritional effects and can be more readily absorbed.

Based on the molecular structure and origin of currently known tannins and their roles in plant life, tannins are defined as polyphenolic secondary metabolites of higher plants, and they are either galloyl esters or their derivatives, in which galloyl moieties or their derivatives are attached to a variety of polyol, catechin, and triterpenoid cores, or they are oligomeric and polymeric proanthocyanidins that can possess different interflavanyl coupling and substitution patterns (3). Gallotannins are those in which galloyl units or their metadepsidic derivatives are bound to diverse polyol, catechin, or triterpenoid units. Upon hydrolysis by acids, bases, or certain enzymes, gallotannins yield glucose and gallic acid (4).

Though tannins have toxic effects on various organisms, some microorganisms are resistant to tannins and have the ability to degrade them into oligomeric tannins and other useful derivatives, such as gallic acid or pyrogallol. Gallotannins are degraded by some bacteria, fungi, and yeasts, which can only hydrolyze the galloyl residues of galloyl esters of tannins. Tannin acyl hydrolase (EC 3.1.1.20), commonly known as tannase, catalyzes the hydrolysis of the galloyl ester bond of tannins. Tannase belongs to the superfamily of esterases. Since its discovery, tannase has found wide applications in the food, feed, beverage, pharmaceutical, and chemical industries (5). Despite the extensive interest and long history of the study of tannase, there is surprisingly little knowledge about the enzyme at the molecular level, which has become one of the critical factors that limit the large-scale application of

tannase. To our knowledge, the only bacterial tannases that have been analyzed genetically are those from *Staphylococcus lugdunensis* (6), *Lactobacillus plantarum* (7, 8), and *Enterobacter* sp. (9). In addition, *L. plantarum* tannase has been biochemically and structurally characterized (7, 8, 10).

L. plantarum is a lactic acid bacterial species that is most frequently encountered in the fermentation of plant materials where tannins are abundant. These plant fermentations include several food and feed products, e.g., olives, grape must, and a variety of vegetable fermentation products. Among food lactic acid bacteria, strains from the *L. plantarum* group possess tannase activity (11–13). The biochemical pathway for the degradation of tannins by *L. plantarum* involves the action of a tannase and a gallate decarboxylase to decarboxylate the gallic acid formed by tannase action (14–16). The *L. plantarum* genes encoding tannase (*tanB$_{Lp}$*, formerly called *tanLp1*) (7) and gallate decarboxylase (*lpdBCD*) (16) involved in tannin degradation have been identified. However, an additional putative *L. plantarum* tannase sequence has been annotated in the genome of an *L. plantarum* strain. In this work, we have characterized the biochemical properties of this novel tannase. The presence of this tannase was analyzed among *L. plantarum* strains. Finally, the relative expression of both tannase genes under methyl gallate exposure was studied.

MATERIALS AND METHODS

Strains and growth conditions. A total of 29 strains of *L. plantarum* were used in this study. *L. plantarum* strains WCFS1, NC8, and LPT 57/1 were kindly provided by M. Kleerebenzem (NIZO Food Research, The Netherlands), L. Axelsson (Norwegian Institute of Food, Fisheries and Aquaculture Research, Norway), and J. L. Ruíz-Barba (Instituto de la Grasa, CSIC; Spain), respectively. Seven strains were provided by the Spanish Type Culture Collection (CECT): *L. plantarum* CECT 220 (ATCC 8014), CECT 221 (ATCC 14431), CECT 223, CECT 224, CECT 749 (ATCC 10241), CECT 4645 (NCFB 1193), and the type strain *L. plantarum* subsp. *plantarum* CECT 748^T (ATCC 14917; DSMZ 20174). Seven strains were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ): *L. plantarum* DSM 1055, DSM 2648, DSM 10492, DSM 12028, DSM 13273, DSM 20246, and the type strain of *L. plantarum* subsp. *argentoratensis* DSM 16365^T. Eleven strains were isolated from grape must or wines of different wine-producing areas of Spain over the period from 1998 to 2001: *L. plantarum* RM28, RM31, RM34, RM35, RM38, RM39, RM40, RM41, RM71, RM72, and RM73 [17]. The *L. plantarum* strains were routinely grown in de Man, Rogosa, and Sharpe medium (MRS) adjusted to pH 6.5 and incubated at 30°C. For the degradation assay, the *L. plantarum* strains were cultivated in a modified basal and defined medium described previously for *L. plantarum* [18]. The basal medium was modified by the replacement of glucose by galactose. This defined medium was used to avoid the presence of phenolic compounds included in nondefined media. The sterilized modified basal medium was supplemented with filter-sterilized tannic acid (1 mM). The *L. plantarum*-inoculated medium was incubated in darkness without shaking at 30°C for 10 days. Incubated medium with cells and without phenolic compound and incubated medium without cells and with phenolic compounds were used as controls. The phenolic products were extracted from the supernatants twice with ethyl acetate (one-third of the reaction volume).

Escherichia coli DH10B was used for all DNA manipulations. *E. coli* BL21(DE3) was used for expression in the pURI3-TEV vector [19]. *E. coli* strains were cultured in Luria-Bertani (LB) medium at 37°C and 140 rpm. When required, ampicillin was added to the medium at a concentration of 100 μg/ml.

PCR detection of tannase-encoding genes. Genes encoding *L. plantarum* tannases (*tanA$_{Lp}$* and *tanB$_{Lp}$*) were amplified by PCR using chromosomal DNA from several lactic acid bacterial strains. The *tanB$_{Lp}$* gene (1.4 kb) was amplified by using primers 951 (5′-TGATGCTGACTGGCT GGTGC) and 952 (5′-GCACAAGCCATCAATCCAGG). Oligonucleotides 953 (5′-CCTGATGAGTGGTTTGTTAG) and 954 (5′-CTTGCGTT CTGCTTCGGTATG) were used to amplify the *tanA$_{Lp}$* gene (1.8 kb). The reactions were performed in a Mastercycler personal thermal cycler (Eppendorf), using 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 1 min, and extension at 72°C for 30 s. The amplified fragments were resolved in agarose gels.

RNA isolation, real-time (RT)-PCR, and qPCR. For RNA isolation, *L. plantarum* MRS cultures were grown to an optical density at 600 nm (OD$_{600}$) of 1 and then supplemented with methyl gallate at 15 mM final concentration. As a control, RNA was also isolated from cultures not supplemented with methyl gallate. After 10 min of incubation, the cultures were immediately processed for RNA extraction as previously described [20]. After DNase I treatment, the absence of DNA from the RNA samples was verified by PCR. The DNA-free RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. From the DNA obtained, quantitative gene expression was analyzed in an AbiPrism 7500 Fast Real Time PCR system (Applied Biosystems). Specific primer pairs were designed with the Primer Express 3.0 program to amplify internal regions of tannase genes. Oligonucleotides 1168 (5′-TGCGCTACCGTG GGATATTC) and 1169 (5′-AATCCAGGAAAATAAATCGCCTAA) were used to amplify 64 bp of *tanB$_{Lp}$*, and primers 1286 (5′-AAAGCAAGCTA CGCCAAAGC) and 1287 (5′-CCCTGGGCATCCGTCTTC) were used to

amplify a 56-bp fragment of *tanA$_{Lp}$*. The expression level of the endogenous control gene (*ldh*) was assayed with primers 918 (5′-AACCGCGAC AATGTTTTGATT) and 919 (5′-TTGTGAACGGCAGTTTCAGTGT). Amplifications were performed in triplicate. All quantitative-PCR (qPCR) assays amplified a single product, as determined by melting-curve analysis and by electrophoresis. A standard curve was plotted with cycle threshold (C_t) values obtained from amplification of known quantities of cDNA and used to determine the efficiency (E) as follows: $E = 10^{-1/\text{slope}}$. In order to measure *L. plantarum* gene expression, amplification of the endogenous control gene was performed simultaneously, and its relative expression was compared with that of the target gene. Relative expression levels were calculated with the Applied Biosystems 7500 Fast System relative quantification software using the *L. plantarum ldh* gene as the endogenous gene and growth in the absence of methyl gallate as the growth condition calibrator.

Expression and purification of TanA$_{Lp}$ from *L. plantarum* ATCC 14917^T. As a peptide signal was predicted in the TanA$_{Lp}$ sequence, the gene *tanA$_{Lp}$* in the locus HPREF0531_11477 from *L. plantarum* ATCC 14917^T was PCR amplified, but lacking the 22-amino-acid peptide signal. The gene was amplified with Prime Star HS DNA polymerase (TaKaRa) by using the primers 805 (5′-*GGTGAAAACCTGTATTTCCAGGGC*gcttg cggacactccgaaacgaaga) and 637 (5′-*ATCGATAAGCTTAGTTAGCTAT*ta cttcaagctcttgttgacccactta) (the nucleotides pairing with the expression vector sequence are in italics, and the nucleotides pairing with the *tanA$_{Lp}$* gene sequence are in lowercase). The gene was cloned into the pURI3-TEV vector, which encodes expression of a leader sequence containing a six-histidine affinity tag. The purified PCR product was then inserted into the pURI3-TEV vector by using a restriction enzyme- and ligation-free cloning strategy [19]. *E. coli* DH10B cells were transformed, and the recombinant plasmids were isolated. Those containing the correct insert were used for transformation of *E. coli* BL21(DE3) cells.

E. coli cells carrying the recombinant pURI3-TEV-TanA$_{Lp}$ plasmid were grown at 37°C in LB medium containing ampicillin (100 μg/ml) and induced by adding 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After induction, the cells were grown at 22°C for 20 h and harvested by centrifugation (7,500 × g for 15 min at 4°C). The cells were resuspended in 50 mM sodium phosphate buffer, pH 7.0, containing 300 mM NaCl. Crude extracts were prepared by French press lysis of cell suspensions (three cycles at 1,100 lb/in^2). The lysates were centrifuged at 17,400 × g for 40 min at 4°C. The supernatant obtained was filtered through a 0.22-μm filter (Millipore) and gently mixed for 20 min at room temperature with 1 ml Talon resin (Clontech). The resin was washed with 50 mM sodium phosphate buffer, pH 7.0, containing 300 mM NaCl and 10 mM imidazole. The recombinant His$_6$-tagged protein was eluted with 50 mM sodium phosphate, pH 7.0, containing 300 mM NaCl and 150 mM imidazole. The eluted His$_6$-tagged TanA$_{Lp}$ was dialyzed overnight at 4°C against 50 mM sodium phosphate buffer, pH 7.0, containing 300 mM NaCl. The purity of the enzyme was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in Tris-glycine buffer.

Tannase activity assay. Since tannase catalyzes the hydrolysis of the galloyl ester linkage liberating gallic acid, the activity of tannase could be measured by estimating the gallic acid formed due to enzyme action [21]. A method specific for the detection of gallic acid could be used for a reliable quantification of tannase activity. Inoue and Hagerman described a rhodanine assay for determining free gallic acid [22]. Rhodanine reacts only with gallic acid and not with galloyl esters of other phenolics. Rhodanine reacts with the vicinal hydroxyl groups of gallic acid to give a red complex with maximum absorbance at 520 nm. Since the rhodanine assay using commercial tannic acid as the substrate gives high absorbance values due to small amounts of free gallic acid present in the preparation, methyl gallate was used as the substrate.

Gallic acid analysis in reactions was determined in triplicate by using the following assay. TanA$_{Lp}$ (10 μg) in 700 μl of 50 mM phosphate buffer, pH 6.5, was incubated with 40 μl of 25 mM methyl gallate (1 mM final concentration) for 5 min at 37°C. After incubation, 150 μl of a methanolic

rhodanine solution (0.667% rhodanine in 100% methanol) was added to the mixture. After 5 min of incubation at 30°C, 100 μl of 0.5 M KOH was added. The absorbance at 520 nm was measured on a spectrophotometer. A standard curve using gallic acid concentrations ranging from 0.125 to 1 mM was prepared. One unit of tannase activity was defined as the amount of enzyme required to release 1 μmol of gallic acid per minute under standard reaction conditions.

Biochemical characterization of TanA$_{Lp}$. Activities of TanA$_{Lp}$ from *L. plantarum* ATCC 14917^T were measured at 4, 20, 30, 37, 45, 55, and 65°C to determine the optimal temperature for enzymatic activity. The optimum pH value for tannase activity was determined by studying its pH dependence within the pH range between 3 and 10. Acetic acid-sodium acetate buffer was used for pH 3 to 5, citric acid-sodium citrate buffer for pH 6, sodium phosphate buffer for pH 7, Tris-HCl buffer for pH 8, glycine-NaOH buffer for pH 9, and sodium carbonate-bicarbonate for pH 10. A 100 mM concentration was used in all the buffers. The rhodanine assay was used for the optimal pH characterization of TanA$_{Lp}$. Since the rhodanine-gallic acid complex forms only under basic conditions, after the enzymatic degradation of methyl gallate, KOH was added to the reaction mixture to ensure that the same pH value (pH 11) was achieved in all samples assayed.

For temperature stability measurements, TanA$_{Lp}$ was incubated in 50 mM phosphate buffer, pH 6.5, at 22, 30, 37, 45, 55, and 65°C for 15 min, 30 min, and 1, 2, 5, and 18 h. After incubation, the residual activity was measured as described above.

To test the effects of metals and ions on the activity of TanA$_{Lp}$, the enzymatic activity was measured in the presence of different additives at a final concentration of 1 mM. The additives analyzed were MgCl$_2$, KCl, CaCl$_2$, HgCl$_2$, ZnCl$_2$, Triton X-100, urea, Tween 80, EDTA, dimethyl sulfoxide (DMSO), and β-mercaptoethanol. All the determinations were done in triplicate.

TanA$_{Lp}$ substrate specificity analysis by HPLC. The activity of TanA$_{Lp}$ against 21 potential substrates was analyzed. The substrates assayed were gallic esters (methyl gallate, ethyl gallate, propyl gallate, and lauryl gallate), benzoic esters (methyl benzoate and ethyl benzoate), hydroxybenzoic esters (methyl 4-hydroxybenzoate, ethyl 4-hydroxybenzoate, propyl 4-hydroxybenzoate, and butyl 4-hydroxybenzoate), vanillic ester (methyl vanillate), dyhydroxybenzoic esters (methyl 2,4-dihydroxybenzoate, ethyl 3,4-dihydroxybenzoate or protocatechuic acid ethyl ester, and ethyl 3,5-dihydroxybenzoate), gentisic ester (methyl gentisate), salicylic ester (methyl salicylate), and ferulic esters (ferulic methyl ester and ferulic ethyl ester). Tannic acid and epigallocatechin gallate were also assayed as potential substrates. Tannase (50 μg), in phosphate buffer, pH 6.0 (50 mM), and CaCl$_2$ (1 mM), was incubated at 37°C in the presence of the substrate (1 mM). As controls, phosphate buffers containing the reagents but not the enzyme were incubated under the same conditions. The reaction products were extracted twice with ethyl acetate; the solvent fractions were filtered through a 0.45-μm polyvinylidene difluoride (PVDF) filter and analyzed by high-performance liquid chromatography with diode array detection (HPLC-DAD). A Thermo chromatograph (Thermo Electron Corporation, Waltham, MA, USA) equipped with a P4000 SpectraSystem pump, an AS3000 autosampler, and a UV6000LP photodiode array detector were used. A gradient of solvent A (water-acetic acid, 98:2 [vol/vol]) and solvent B (water-acetonitrile-acetic acid, 78:20:2 [vol/vol/vol]) was applied to a reversed-phase Nova-pack C$_{18}$ cartridge (25 cm by 4.0-mm inside diameter [i.d.]; 4.6-μm particle size) at room temperature as follows: 0 to 55 min, 80% B linear, 1.1 ml/min; 55 to 57 min, 90% B linear, 1.2 ml/min; 57 to 70 min, 90% B isocratic, 1.2 ml/min; 70 to 80 min, 95% B linear, 1.2 ml/min; 80 to 90 min, 100% linear, 1.2 ml/min; 100 to 120 min, washing, 1.0 ml/min; and reequilibration of the column under the initial gradient conditions. Samples were injected onto the cartridge after being filtered through a 0.45-μm PVDF filter. Detection of the substrates and the degradation compounds was performed spectrophotometrically by scanning from 220 to 380 nm. The identification of degradation compounds was carried out by comparing the retention times

and spectral data of each peak with those of standards from commercial suppliers or by LC-DAD– electrospray ionization-mass spectrometry (ESI-MS).

Sequence data analysis. A homology search with finished and unfinished microbial genome databases was performed with the BLAST algorithm at the National Center for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi). Multiple alignments were made using the Clustal W2 Program (http://www.ebi.ac.uk/Tools/msa/clustalw2/) on the EBI site after retrieval of sequences from the GenBank and Swiss-Prot databases. Computer promoter predictions were carried out at the Internet site (http://www.fruitfly.org/seq_tools/promoter.html). pI and molecular weight (MW) were analyzed on EXPASY (http://web.expasy.org/compute_pi/), signal peptide cleavage sites were analyzed on the SignalP 4.1 server site (http://www.cbs.dtu.dk/services/SignalP/), and predicted transcription terminators were analyzed at the ARNold site (http://rna.igmors.u-psud.fr/toolbox/arnold/index.php#Results).

RESULTS

Presence of a putative novel tannase-encoding gene in several *L. plantarum* strains. Homology searches in genome databases allowed us to find the locus HMPREF0531_11477 in the *L. plantarum* ATCC 14917^T genome, which encodes a 626-amino-acid-residue protein annotated as "tannase," defined here as TanA$_{Lp}$. Interestingly, multiple amino acid sequence alignments of TanA$_{Lp}$ from *L. plantarum* ATCC 14917^T with TanA$_{Sl}$ tannase from *S. lugdunensis* and TanB$_{Lp}$ tannase from *L. plantarum* revealed high sequence identity to TanA$_{Sl}$ (50%) and much lower identity to TanB$_{Lp}$ (27%) (see Fig. S1 in the supplemental material). Moreover, TanA$_{Lp}$ shares additional features with TanA$_{Sl}$ from *S. lugdunensis*. Both are 67-kDa proteins and have alkaline isoelectric points (9.54 for TanA$_{Sl}$ and 9.94 for TanA$_{Lp}$), and they present predicted signal peptides. On the other hand, TanB$_{Lp}$ is a 50-kDa protein with an isoelectric point of 6.06, and it does not possess a signal peptide.

Based on the recently described crystal structure of TanB$_{Lp}$ (10), the conserved sequence motif Gly-X-Ser-X-Gly typical of serine hydrolases could be easily identified in TanA$_{Lp}$ from *L. plantarum* ATCC 14917^T (Gly-215 to Gly-219 in TanA$_{Lp}$). From the catalytic triad identified in the structure (Ser-163, His-451, and Asp-419 in TanB$_{Lp}$), only serine and histidine residues are conserved in TanA$_{Lp}$ as well as in TanA$_{Sl}$ (see Fig. S1 in the supplemental material), with Asp-419 being replaced by a Gln residue in both proteins. On the other hand, the residues which make contacts with the three hydroxyl groups of gallic acid (Asp-421, Lys-343, and Glu-357 in TanB$_{Lp}$) are conserved in both TanA$_{Lp}$ and TanA$_{Sl}$ (see Fig. S1 in the supplemental material).

The genomes of 10 *L. plantarum* strains are currently available. Analyses of these genomes revealed that a copy of the *tanA$_{Lp}$* gene is present only in *L. plantarum* ATCC 14917^T and *L. plantarum* NC8 and is not found in the rest (*L. plantarum* WCFS1, JDM1, ST-III, 16, P-8, IPLA 88, UCMA 3037, and ZJ316). In the ATCC 14917^T and NC8 strains, *tanA$_{Lp}$* is located between the genes *nox3* (encoding a NADH oxidase [accession number EFK29315]) and *dapE1* (encoding succinyl-diaminopimelate desuccinylase [accession number EFK29313]) (Fig. 1). The intergenic region between these genes is 121 bp long in the *L. plantarum* strains devoid of *tanA$_{Lp}$*; however, in the strains possessing *tanA$_{Lp}$*, this region is 2,515 or 2,516 bp long in NC8 and ATCC 14917^T, respectively. In *L. plantarum* ATCC 14917^T, this region encodes a 626-amino-acid-residue protein (TanA$_{Lp}$), which is preceded by a putative
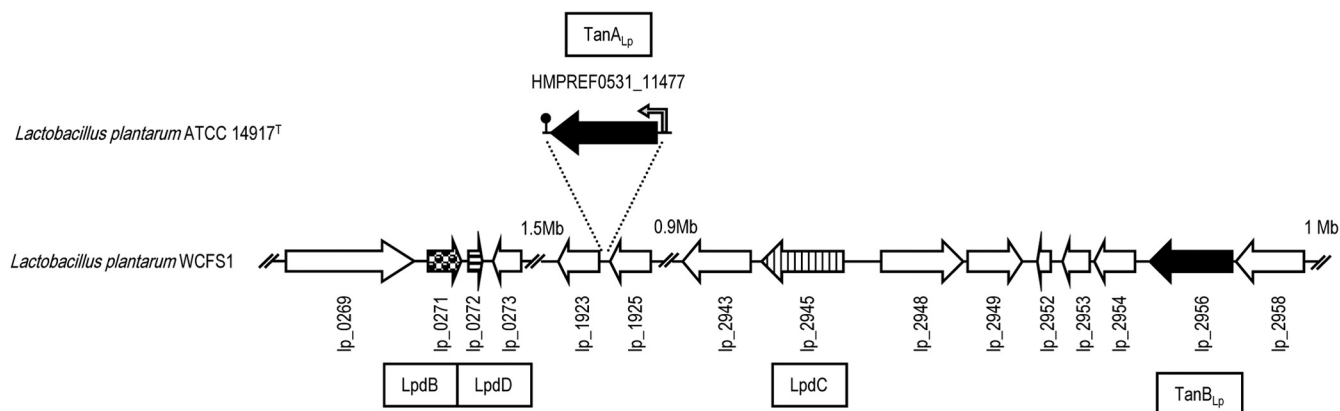
FIG 1 Genetic organization of the *L. plantarum* WCFS1 chromosomal region containing the gallate decarboxylase- and tannase-encoding genes (accession no. NC_004567; positions 243093 to 252815, 1743368 to 1746325, and 2618290 to 2635122). The insertion of the *tanA_{Lp}* gene in *L. plantarum* ATCC 14917^T is also represented (accession no. ACGZ02000013.1; positions c/26013 to 27893). The arrows represent genes. Genes coding for putative tannase proteins are represented by black arrows. Genes encoding gallate decarboxylase subunits (Lpd, LpdC, and LpdD) are also shown. The locations of a putative *tanA_{Lp}* promoter (vertical bent arrow) and transcription terminator region (ball and stick) are also indicated.

promoter, as revealed by sequence analysis. In turn, a putative transcription terminator site follows the stop codon, where a possible stem-loop structure is predicted, which would start 45 nucleotides downstream from the TAA stop codon and would have a 19-base stem and a 22-base loop. This structure may serve as a terminator for transcription (Fig. 1). The *L. plantarum* NC8 sequence is identical to that of ATCC 14917^T with the exception of a G deletion, which produces a frameshift from Gln-529 to the end of TanA_{Lp} (see Fig. S2 in the supplemental material).

In order to know the extent of the tannase genes among *L. plantarum* strains, the presence of the *tanB_{Lp}* and *tanA_{Lp}* genes was studied in 29 *L. plantarum* strains isolated from different sources. To determine the presence of both genes, chromosomal DNA was extracted and PCR amplified. DNA fragments of 1.4 or 1.8 kb from *tanB_{Lp}* or *tanA_{Lp}*, respectively, were PCR amplified using oligonucleotides designed on the basis of the *L. plantarum* ATCC 14917^T sequence. All the *L. plantarum* strains analyzed gave the corresponding *tanB_{Lp}* amplicon (data not shown), which indicates that TanB_{Lp} is generally present among *L. plantarum* strains, as described previously (7). Apart from ATCC 14917^T and NC8, two additional strains also possessed a copy of the *tanA_{Lp}* gene, namely, *L. plantarum* CECT 749 and RM35 (data not shown). Since the NC8 *tanA_{Lp}* copy is truncated, the complete sequences of the CECT 749 and RM35 strains were determined. Alignment of the resulting TanA_{Lp} amino acid sequences revealed one substitution in the CECT 749 protein (Ala-107 to Asp-107) and two in the RM35 strain (Arg-563 to Lys-563, and Arg-565 to Gln-565) (see Fig. S2 in the supplemental material). It is noteworthy that these two mutated arginine residues are part of a motif (WRIR) conserved in all tannases (see Fig. S1 in the supplemental material).

Extracellular tannase activity on *L. plantarum* strains. Since the sequences of the four *tanA_{Lp}* copies present in the *L. plantarum* strains analyzed here differ, an activity assay was done to determine the functionality of the resulting coded proteins. In this regard, the presence of a putative signal peptide indicated that TanA_{Lp} could be an extracellular protein, and in fact, an extracellular tannase produced by an *L. plantarum* strain has been reported (23). *L. plantarum* WCFS1 and the four strains possessing a *tanA_{Lp}* copy were grown in a basal medium containing 1 mM

tannic acid for 10 days. Tannic acid was chosen because it is a complex gallotannin unable to pass into the cell to be degraded by intracellular TanB_{Lp} tannase. As a control, the medium was incubated under the same conditions. From the *L. plantarum* culture, the cells were pelleted, and the tannic acid in the supernatant was extracted and analyzed by HPLC. Figure 2 shows that most of the chromatograms were similar to the control, and only small variations were observed among them. However, *L. plantarum* ATCC 14917^T showed a chromatogram that clearly indicated hydrolysis of the high-molecular-weight tannins, suggesting the presence of an active extracellular tannase only in this strain.

Relative expression of *L. plantarum* tannase genes under methyl gallate exposure. As tannase is involved in tannin degradation, the relative expression of both tannase-encoding genes under methyl gallate exposure was studied. Strain WCFS1 was analyzed as a model strain with only one tannase enzyme and strain ATCC 14917^T as a model strain having two different active tannase proteins. *L. plantarum* cultures were induced for 10 min by the presence of 15 mM methyl gallate as a potential tannase substrate. The gene expression levels obtained were substantially different between the two tannase-encoding genes (data not shown), indicating the presence of two different expression patterns for the proteins. The *tanA_{Lp}* gene, only present in *L. plantarum* ATCC 14917^T, showed an expression level not affected by the presence of its substrate, methyl gallate. However, the *tanB_{Lp}* gene expression profiles were affected. In both *L. plantarum* strains, the presence of 15 mM methyl gallate induces about a 3-fold increase in the expression of the *tanB_{Lp}* gene.

Biochemical properties of TanA_{Lp} from *L. plantarum* ATCC 14917^T. Of the *L. plantarum* strains possessing two tannase enzymes, only *L. plantarum* ATCC 14917^T showed extracellular tannase activity; therefore, TanA_{Lp} from this strain was biochemically characterized. The *tanA_{Lp}* gene lacking the 22-amino-acid peptide signal sequence from *L. plantarum* ATCC 14917^T was expressed in *E. coli* under the control of an inducible promoter. Cell extracts were used to detect the presence of overproduced proteins by SDS-PAGE analysis. Control cells containing the pURI3-TEV vector plasmid did not show protein overexpression; in addition, no tannase activity was observed in this control extract. However, an
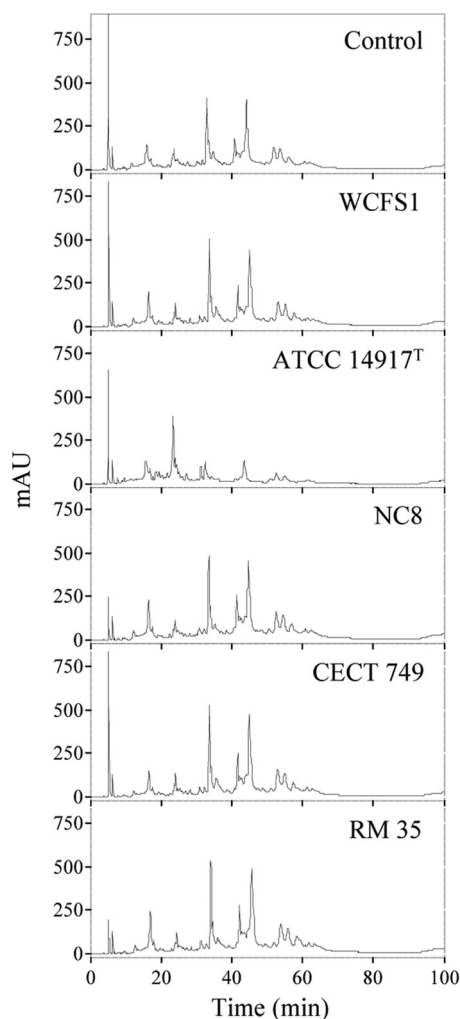
FIG 2 HPLC analysis of tannic acid degradation by *L. plantarum* cultures. Modified basal medium containing 1 mM tannic acid was inoculated with *L. plantarum* strains (WCFS1, ATCC 14917^T, NC8, CECT 749, and RM 35) and incubated for 10 days. A noninoculated control medium was incubated under the same conditions. Detection was performed at 280 nm. AU, absorbance units.

FIG 3 Purification of *L. plantarum* TanA$_{Lp}$ protein. Shown are SDS-PAGE analysis of the expression and purification of His$_6$-TanA$_{Lp}$ and analysis of soluble cell extracts of IPTG-induced *E. coli* BL21(DE3)(pURI3-TEV) (lane 1) and *E. coli* BL21(DE3)(pURI3-TEV-TanA$_{Lp}$) (lane 2) or fractions eluted after His affinity resin (lanes 3 to 7). The arrow indicates the overproduced and purified protein. The 12.5% gel was stained with Coomassie blue. Molecular mass markers are located on the left (SDS-PAGE Standards; Bio-Rad).

overproduced protein with an apparent molecular mass of around 67 kDa was observed in cells harboring pURI3-TEV-TanA$_{Lp}$ (Fig. 3). Since the cloning strategy yielded a His-tagged protein variant, *L. plantarum* pURI3-TEV-TanA$_{Lp}$ could be purified on an immobilized metal affinity chromatography (IMAC) resin. However, unexpectedly, the protein was scarcely purified (2.71 mg/liter), with most of the protein not being bound to the resin.

The *L. plantarum* ATCC 14917^T TanA$_{Lp}$ enzyme, partially purified by the affinity resin, was biochemically characterized. Tannase activity was determined by using methyl gallate as the substrate. The specific activities of TanA$_{Lp}$ and TanB$_{Lp}$ (taken as references) were determined by the rhodanine assay. TanA$_{Lp}$ has a specific activity of 39 U/mg, whereas TanB$_{Lp}$ has 404 U/mg.

Figure 4 shows the optimum temperature and pH and the thermal stability of TanA$_{Lp}$ as determined by the rhodanine assay, with methyl gallate as the substrate. TanA$_{Lp}$ displays optimal activity within the 20 to 30°C temperature range and an optimal pH around 6. Figure 4D shows the effects of various additives (1 mM
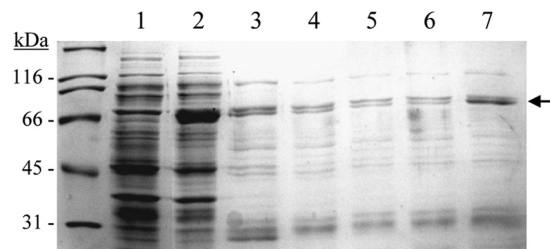
final concentration) on the enzymatic activity of TanA$_{Lp}$. CaCl$_2$ greatly increased and HgCl$_2$ completely inhibited TanA$_{Lp}$ activity. In relation to TanA$_{Lp}$ substrates, similarly to TanB$_{Lp}$, only the esters derived from gallic and protocatechuic acids were hydrolyzed (see Fig. S3 in the supplemental material). It seems that other cinnamic acids without hydroxyl groups and with substituents other than —H or —OH at position 2 were not metabolized by TanA$_{Lp}$ or by TanB$_{Lp}$. Regarding the aliphatic alcohol constituent of the ester bond, a lauryl substituent could not be effectively hydrolyzed by TanA$_{Lp}$ (see Fig. S3 in the supplemental material).

DISCUSSION

Although tannase activity has been extensively described in *L. plantarum* strains (12–14), the first bacterial tannase genetically identified was TanA$_{Sl}$ from *S. lugdunensis* (6), and then, by sequence comparison, TanB$_{Lp}$ (Lp_2956) from *L. plantarum* was later identified (7). The first complete genome of an *L. plantarum* strain was from the WCFS1 strain. However, at present, the sequences of several *L. plantarum* strains are publicly available. In *L. plantarum* ATCC 14917^T, the locus HMPREF0531_11477 was annotated as tannase and designated TanA$_{Lp}$. TanA$_{Lp}$ from *L. plantarum* ATCC 14917^T showed higher sequence identity to TanA$_{Sl}$ from *S. lugdunensis* (50%) than to *L. plantarum* TanB$_{Lp}$ tannase (27%). Despite the low identity to TanB$_{Lp}$, TanA$_{Sl}$ and TanA$_{Lp}$ shared TanB$_{Lp}$ motifs involved in tannase activity (10). Therefore, structural data also suggest that TanA$_{Lp}$ from *L. plantarum* ATCC 14917^T could be an active tannase.

When the presence of both tannases was studied in 36 *L. plantarum* strains, it was observed that a copy of the *tanB$_{Lp}$* gene was present in all the analyzed strains, whereas *tanA$_{Lp}$* was present in only four *L. plantarum* strains (ATCC 14917^T, NC8, CECT 749, and RM35). The analysis of the TanA$_{Lp}$ protein sequences from these four strains revealed that, compared to TanA$_{Lp}$ from *L. plantarum* ATCC 14917^T, TanA$_{Lp}$ from CECT 749 and RM35 possessed amino acid substitutions in a motif conserved in all tannases. Moreover, the sequence of *tanA$_{Lp}$* from *L. plantarum* NC8 posses a frameshift that produces a truncated protein lacking 2 of the 3 residues of the catalytic triad (residues equivalent to His-451 and Asp-419 in TanB$_{Lp}$) and Asp-421, which make contacts with a hydroxyl group of gallic acid.

From the sequence analysis, TanA$_{Lp}$ from *L. plantarum* ATCC 14917^T seems to be the only TanA$_{Lp}$ tannase in which the residues important for activity are conserved. In order to verify this hy-
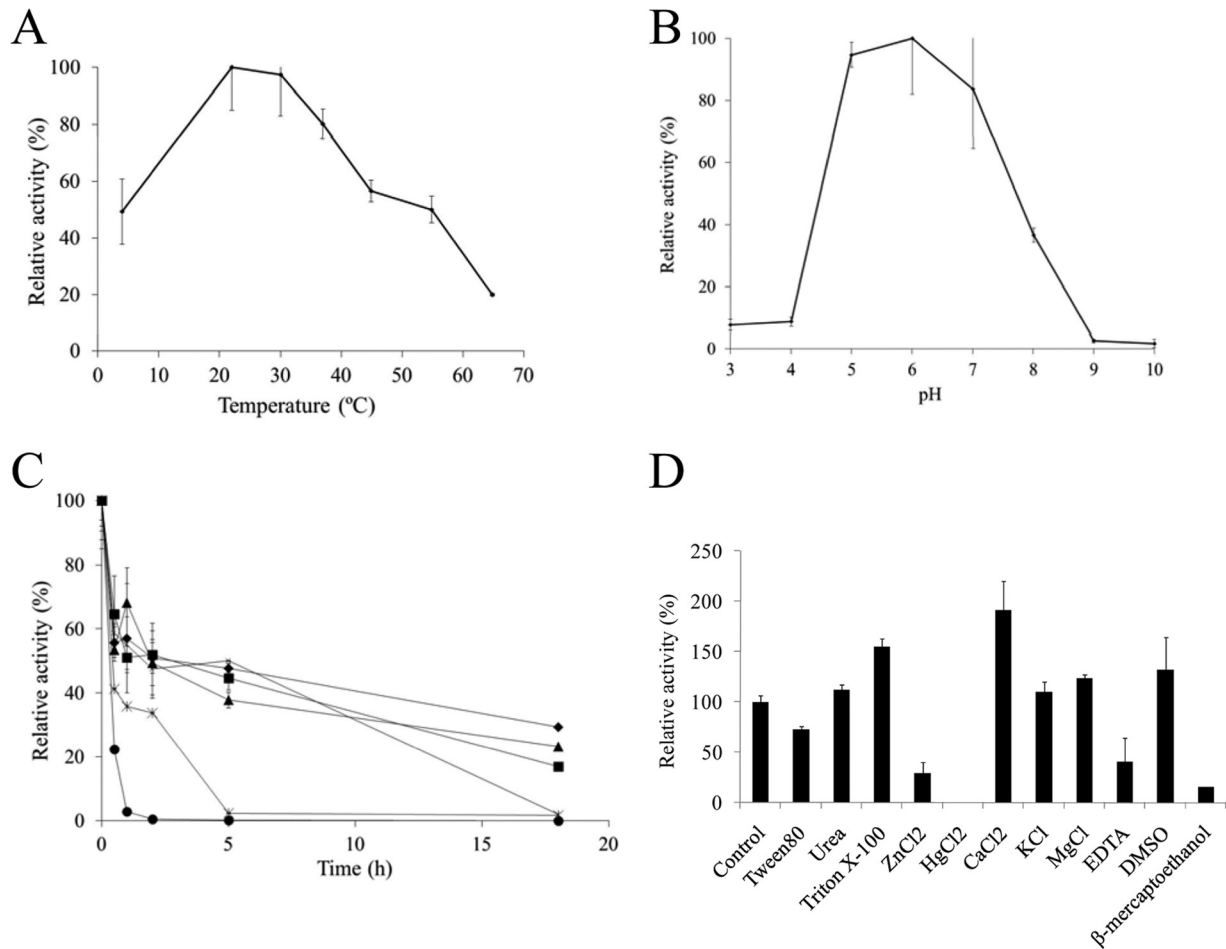
FIG 4 Some biochemical properties of TanA$_{Lp}$ protein. (A) Relative activity of TanA$_{Lp}$ versus temperature. (B) Relative activity versus pH. (C) Thermal stability of TanA$_{Lp}$ after preincubation at 22°C (diamonds), 30°C (squares), 37°C (triangles), 45°C (×), 55°C (stars), and 65°C (circles) in phosphate buffer (50 mM; pH 6.5). At the indicated times, aliquots were withdrawn and analyzed as described in Materials and Methods. The experiments were done in triplicate. The mean values and standard errors are shown. The observed maximum activity was defined as 100%. (D) Relative activity of TanA$_{Lp}$ after incubation with 1 mM concentrations of different additives. The activity of the enzyme incubated in the absence of additives was defined as 100%.

pothesis, and taking into account that TanA$_{Lp}$ seems to be an extracellular tannase, strains possessing a *tanA$_{Lp}$* copy (ATCC 14917^T, NC8, CECT 749, and RM35) were grown in the presence of tannic acid, a complex gallotannin unable to pass into the cell to be degraded by intracellular TanB$_{Lp}$ tannase. As expected, the presence of an active extracellular tannase was observed only in *L. plantarum* ATCC 14917^T. In this strain, in addition to the functionality of TanA$_{Lp}$, the functionality of TanB$_{Lp}$ was previously demonstrated (8). As the strain possesses two tannase proteins able to hydrolyze gallotannins, the expression of these proteins was studied under methyl gallate exposure. The presence of the substrate methyl gallate did not affect the expression of *tanA$_{Lp}$*; however, it increased the expression of *tanB$_{Lp}$*. This expression behavior allows the assumption that *tanB$_{Lp}$* encodes an inducible tannase in *L. plantarum* ATCC 14917^T, as previously observed in the WCFS1 strain under tannic acid challenge (24).

From the results obtained in this study, it seems that the two *L. plantarum* tannases play different physiological roles. These different functions could be partially attributed to their different biochemical properties, such as their reported substrate spectra, which seem to be very similar except that esters having a long

aliphatic alcohol were not effectively hydrolyzed by TanA$_{Lp}$. Moreover, TanA$_{Lp}$ from *L. plantarum* ATCC 14917^T has a specific activity of 39 U/mg, 10 times lower than the specific activity calculated for TanB$_{Lp}$ (404 U/mg) from the same strain. In addition, the optimal temperature and pH for TanA$_{Lp}$ (20 to 30°C and pH 6) differed from those described for TanB$_{Lp}$ (40°C and pH 7 to 8) (7, 8). Interestingly, the optimal activity of TanA$_{Lp}$ is similar to the optimal tannase activity shown by cell extracts from *L. plantarum* ATCC 14917^T (25). It is noteworthy that *L. plantarum* ATCC 14917^T cell extracts were obtained from cultures grown in a medium devoid of possible tannase inducers, on which TanB$_{Lp}$ tannase could not be induced. Therefore, it seems that in the absence of a substrate, the biochemical characteristics of tannase activity shown by cell extracts of *L. plantarum* ATCC 14917^T, containing both tannase genes, are more similar to those exhibited by TanA$_{Lp}$ than to those of TanB$_{Lp}$. This could indicate that, in the absence of a substrate, TanA$_{Lp}$ activity predominates in *L. plantarum* strains having two tannase enzymes. The gene expression results indicated that TanA$_{Lp}$ is not inducible by the presence of methyl gallate; however, its basal expression level could be enough to be detected in *L. plantarum* cell extracts. In complex tannins, such as

tannic acid, the presence of an extracellular and low-activity TanA$_{Lp}$ tannase in some *L. plantarum* strains could provide an enzymatic activity able to partially degrade tannic acid outside the cell. The less complex tannins originated by TanA$_{Lp}$ action could be able to induce the expression of *tanB$_{Lp}$* and pass into the cell to be degraded by TanB$_{Lp}$. In addition, TanA$_{Lp}$ biochemical properties are more convenient than those from TanB$_{Lp}$ for an extracellular enzyme acting on plant substrates. Temperatures around 20 to 30°C and acidic pH could be environmental conditions present on these plant substrates.

The specific catabolic capacity of *L. plantarum* against gallotannins suggests that they provide a selective advantage to the species for life in environments where compounds of plant origin are abundant. The presence in some *L. plantarum* strains of a second active tannase provides an additional advantage. *L. plantarum* should be able to degrade them with TanA$_{Lp}$ and does not depend on other microorganisms for the initial degradation of these compounds. This second tannase, TanA$_{Lp}$, is an extracellular enzyme able to hydrolyze complex gallotannins, which are unable to pass into the cell to be degraded by TanB$_{Lp}$. Moreover, the presence of TanA$_{Lp}$ provides *L. plantarum* with strains an additional response mechanism to overcome the adverse effects of tannins present in their environment.

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